



Autoantibody Signature in Cardiac Arrest

Editorial, see p 1775

Ange Maguy, PhD
Jean-Claude Tardif, MD
David Busseuil, PhD
Camillo Ribi, MD
Jin Li, MD

BACKGROUND: Cardiac arrest is a tragic event that causes 1 death roughly every 90 seconds worldwide. Survivors generally undergo a workup to identify the cause of arrest. However, 5% to 10% of cardiac arrests remain unexplained. Because cardiac arrhythmias underlie most cardiac arrests and increasing evidence strongly supports the involvement of autoantibodies in arrhythmogenesis, a large-panel autoantibody screening was performed in patients with cardiac arrest.

METHODS: This is an observational, cross-sectional study of patients from the Montreal Heart Institute hospital cohort, a single-center registry of participants. A peptide microarray was designed to screen for immunoglobulin G targeting epitopes from all known cardiac ion channels with extracellular domains. Plasma samples from 23 patients with unexplained cardiac arrest were compared with those from 22 patients with cardiac arrest cases of ischemic origin and a group of 29 age-, sex-, and body mass index-matched healthy subjects. The false discovery rate, least absolute shrinkage and selection operator logistic regression, and random forest methods were carried out jointly to find significant differential immunoglobulin G responses.

RESULTS: The autoantibody against the pore domain of the L-type voltage-gated calcium channel was consistently identified as a biomarker of idiopathic cardiac arrest ($P=0.002$; false discovery rate, 0.007; classification accuracies ≥ 0.83). Functional studies on human induced pluripotent stem cell-derived cardiomyocytes demonstrated that the anti-L-type voltage-gated calcium channel immunoglobulin G purified from patients with idiopathic cardiac arrest is proarrhythmogenic by reducing the action potential duration through calcium channel inhibition.

CONCLUSIONS: The present report addresses the concept of autoimmunity and cardiac arrest. Hitherto unknown autoantibodies targeting extracellular sequences of cardiac ion channels were detected. Moreover, the study identified an autoantibody signature specific to patients with cardiac arrest.

Key Words: arrhythmias, cardiac
■ autoantibodies ■ heart arrest
■ microarray analysis

Sources of Funding, see page 1773

© 2020 The Authors. *Circulation* is published on behalf of the American Heart Association, Inc., by Wolters Kluwer Health, Inc. This is an open access article under the terms of the [Creative Commons Attribution Non-Commercial-NoDerivs](#) License, which permits use, distribution, and reproduction in any medium, provided that the original work is properly cited, the use is noncommercial, and no modifications or adaptations are made.

<https://www.ahajournals.org/journal/circ>

Clinical Perspective

What Is New?

- The present study explored the autoantibody profile of patients with idiopathic and ischemic cardiac arrest compared with healthy control subjects.
- New autoantibodies targeting cardiac ion channels were detected.
- Anti-L-type voltage-gated calcium channel autoantibodies are linked to idiopathic cardiac arrest.
- Anti-TWIK-related potassium channel 1 autoantibodies are found in patients with ischemic cardiac arrest and healthy control subjects.

What Are the Clinical Implications?

- Potential immunoglobulin G biomarkers for cardiac arrest were identified.

Cardiac arrest is a tragic event that causes 1 death roughly every 90 seconds worldwide.^{1,2} Structural heart disease is the main culprit and predisposes the patient to potentially life-threatening ventricular tachyarrhythmias (VTs).^{1,2} In the absence of structural heart disease, the differential diagnosis includes primary electrical disorders (eg, long-QT syndrome, short-QT syndrome, Brugada syndrome, catecholaminergic polymorphic VT).³ Despite the many advances in diagnostic tools ranging from cardiac imaging studies and coronary angiography to genetic testing, 5% to 10% of cardiac arrests remain unexplained.^{2,4–6} In the past decade, emerging data have suggested the involvement of autoantibodies in the pathogenesis of cardiac arrhythmias.⁷ Nevertheless, to the best of our knowledge, an autoantibody profile of cardiac arrest has not been systematically investigated so far in a controlled study. In the search for arrhythmogenic autoantibodies, a large-panel autoantibody screening was performed in patients who survived a cardiac arrest of unknown origin and compared with that from patients with cardiac arrest of ischemic origin and healthy subjects. A special emphasis was placed on autoantibodies targeting cardiac ion channels.

METHODS

The data that support the findings of this study are available from the corresponding author on reasonable request.

Study Design

This study is reported in accordance with the guidelines from the Strengthening the Reporting of Observational Studies in Epidemiology Consortium.⁸ Any member of the Montreal Heart Institute (MHI) community, including patients with or at risk for heart disease, individuals attending the hospital for routine blood tests, family members of patients, and

employees, may participate in this cohort. Between May 2007 and November 2018, among 16 762 participants of the MHI hospital cohort, 74 patients were identified for the present study according to the following selection criteria: survivors of cardiac arrest who underwent a comprehensive cardiac workup, notably a detailed history, ECG, transthoracic echocardiography, cardiac magnetic resonance imaging when indicated, and coronary angiography. Cardiac arrest was defined as “cessation of cardiac activity such that the victim becomes unresponsive, with no normal breathing and no signs of circulation,” in accordance with the current guidelines but including only cardiac arrest resulting from shockable rhythm (ie, documented pulseless ventricular tachycardia or ventricular fibrillation).⁹ The scope of the present study thus involved cases of in-hospital and out-of-hospital cardiac arrest. Patients with autoimmune diseases, immunodeficient conditions, or malignancies were excluded from the study. Twenty-two patients meeting the aforementioned inclusion and exclusion criteria had a cardiac arrest subsequent to myocardial ischemia (referred to as ischemic cardiac arrest) as evidenced by coronary angiography. Twenty-three cases of cardiac arrest were of unknown origin. The patients were classified as having idiopathic cardiac arrest when the cardiac arrest was unexplained and when the patients did not have any structural heart disease (excluded by echocardiography or cardiac magnetic resonance imaging), coronary artery disease (excluded by coronary angiography), or primary electric disorder (negative genetic testing). When appropriate, an epinephrine and procainamide provocation test was performed in these patients but did not unmask any long-QT syndrome, catecholaminergic polymorphic VT, or Brugada syndrome. Targeted genetic testing was performed on the basis of phenotype detection and evaluated in accordance with the guidelines by the American College of Medical Genetics and Genomics.¹⁰ In addition, 29 age-, sex-, and body mass index-matched healthy subjects (referred to as healthy) from the MHI hospital cohort were included in the autoantibody screening. They were considered to be in the healthy group when they had no medical condition, in particular no history of or known cardiac disease and a normal ECG and heart function (preserved left ventricular ejection fraction, no signs of ischemia on exercise electrocardiographic testing, stress echocardiography, or myocardial scintigraphy). All participants provided written informed consent to participate in the MHI hospital cohort. The study protocol was approved by the local ethics committee of the MHI. The study was conducted in accordance with the Declaration of Helsinki and the guidelines of Good Clinical Practice issued by the International Conference on Harmonization.

Antigenic Epitopes

Cardiac ion channels are composed of a number of membrane-spanning, pore-forming proteins. Because the extracellular domains are naturally accessible, they are putative recognition sites for circulating antibodies. Peptides from extracellular domains of cardiac ion channels were therefore exclusively selected, as done previously for the KCNQ1 study.^{11,12} In the present de novo search for autoantibodies, a peptide library consisting of epitopes derived from all existing cardiac ion channels was generated, including the sodium,

calcium, potassium channels, accessory subunits (minimal potassium channel subunit, minimal potassium channel subunit-related peptide), and transient receptor potential channels. Additional antigenic peptides from G-protein-coupled receptors (M2-muscarinic, β_1 - and β_2 -adrenergic receptors) were included because they were described previously in the literature in the context of VT.⁷ To ascertain the antigenicity of the epitope selected, all sequences were analyzed beforehand with the Kolaskar and Tongaonkar method¹³ and with the Support Vector Machine integrated with the tripeptide similarity and propensity scores (<http://sysbio.unl.edu/SVMTriP>).¹⁴

PEPperMAP Immunoglobulin G Profiling Against 100 Selected Peptides

Figure 1A provides an overview of the peptide microarray assay. Plasma samples were collected from all participants and screened for immunoglobulin G (IgG). Based on the peptides of interest, a peptide library (14–20 amino acid lengths) was synthesized (Table 1 in the Data Supplement) and translated into PEPperCHIP Custom Peptide Microarray chips in duplicate (PEPperPRINT GmbH, Heidelberg, Germany). In addition to the selected cardiac ion channel peptide sequences (Table 1 in the Data Supplement), the peptide microarrays were framed by influenza hemagglutinin (YPYDVPDYAG, 15 spots) and polio virus (KEVPALTAVETGAT, 16 spots) control peptides. Peptide microarrays were washed with 1× PBS with 0.05% Tween 20 (3× for 1 minute) after each incubation step. Thirty minutes before the first assay, Rockland blocking buffer MB-070 was applied. Plasma samples were incubated for 16 hours at 4°C (shaking at 140 rpm) at a dilution of 1:30 in incubation buffer (ie, washing buffer with 10% blocking buffer). Goat anti-human IgG (Fc) DyLight800 (1:5000, 45 minutes at room temperature [RT]) served as secondary antibody, and the following control antibodies were used: mouse monoclonal anti-hemagglutinin (12CA5) DyLight800 (1:2000, 45 minutes at RT). High-definition images from the microarrays were then acquired with the LI-COR Odyssey Imaging System (scanning offset, 0.65 mm; resolution, 21 μ m; scanning intensities, 7/7) using wavelengths of 700 nm (red channel, for specific IgG signal quantification) and 800 nm (green channel). For quality control, 1 peptide microarray was incubated with secondary goat anti-human IgG (Fc) DyLight680 antibody (1:5000) and control mouse monoclonal anti-hemagglutinin (12CA5) DyLight800 antibody (1:2000) for 45 minutes at RT to analyze background interactions with the 100 different peptides of the microarray. At scanning intensities of 7/7 (red/green), no background interaction of the secondary or control antibodies with the printed peptides was observed, even on significant increase of brightness and contrast (Figure 1 in the Data Supplement).

Microarray Data Analysis

Data analysts from PEPperPRINT GmbH were blinded to the study design. Microarray image analysis was performed with the PepSlide Analyzer. The software algorithm breaks down fluorescence intensities of each spot into raw foreground and background signal and calculates averaged median foreground intensities and spot-to-spot deviations of spot duplicates. On the basis of the averaged median foreground intensities, an intensity map was generated, and interactions

in the peptide map were highlighted by an intensity color code with red for high and white for low spot intensities. A maximum spot-to-spot deviation of 40% was tolerated; otherwise, the corresponding intensity value was zeroed. To identify the prevailing IgG in the plasma samples, the averaged and corrected intensity values were sorted by decreasing spot intensities. The averaged spot intensities of the assays were further plotted with the plasma samples against the microarray content from right on top to left on bottom in a row-wise manner to visualize overall spot intensities and signal-to-noise ratios. The intensity plots were correlated with peptide and intensity maps and with visual inspection of the microarray scans to identify IgG responses of the samples.

IgG Purification

The plasma samples from all patients with idiopathic cardiac arrest were pooled together. Affinity chromatography using a sulfhydryl-reactive agarose matrix coupled to the DFDNLVAAMMALFTVSTFEG peptide with an N-terminal cysteine (Peptide Specialty Laboratories GmbH, Heidelberg, Germany) was used to purify IgG autoantibodies from the plasma. To this end, the affinity chromatography column (Peptide Specialty Laboratories GmbH) was loaded with the pooled plasma from patients with idiopathic cardiac arrest (diluted 1:1 with 1× PBS) for 4 hours at 4°C with slow rotation. The total effluent was then run through the column twice before 3 washing steps with 1× PBS followed by 2 washing steps with 10 mmol/L Na-phosphate (pH 6.8). The antibodies were eluted from the column with Pierce IgG Elution Buffer (ThermoFisher Scientific, Zug, Switzerland) and collected in 2 mol/L K_2HPO_4 . The solution containing the IgG was then replaced by 1× PBS using diafiltration with the Amicon Ultra 15 filter device (nominal molecular weight limit, 50 kDa; Merck & Cie, Schaffhausen, Switzerland). The IgG concentration of the eluate was then determined with a bicinchoninic acid assay (QuantiPro BCA Assay Kit, Sigma Aldrich GmbH, Buchs, Switzerland).

Human Induced Pluripotent Stem Cell-Derived Cardiomyocyte Culture and Preparation

Human induced pluripotent stem cell-derived ventricular cardiomyocytes (hiPSC-CMCs) were obtained from Ncardia BV (Pluricyte, Leiden, the Netherlands) and cultured according to manufacturer's guidelines. Briefly, cryovials of hiPSC-CMCs were thawed by placing in an incubator at 37°C for 4 minutes before being transferred into a 50-mL tube. The vial was then rinsed with 1 mL Pluricyte Cardiomyocyte Medium, and the cell suspension (drop-wise) was immersed with 4.7 mL Pluricyte Cardiomyocyte Medium. After a centrifugation step at 250g for 3 minutes, the cells were resuspended in fresh Pluricyte Cardiomyocyte Medium and plated with a density of 250.000/cm² on Petri dishes coated with Corning Matrigel (VWR, Dietikon, Switzerland) diluted 1:100 in DMEM/F-12 (STEMCELL Technologies, Cologne, Germany). hiPSC-CMCs were maintained in Pluricyte Cardiomyocyte Medium and incubated at 37°C/5% CO₂. The culture medium was changed every other day. All electrophysiological measurements were performed between days 7 and 14 at RT.

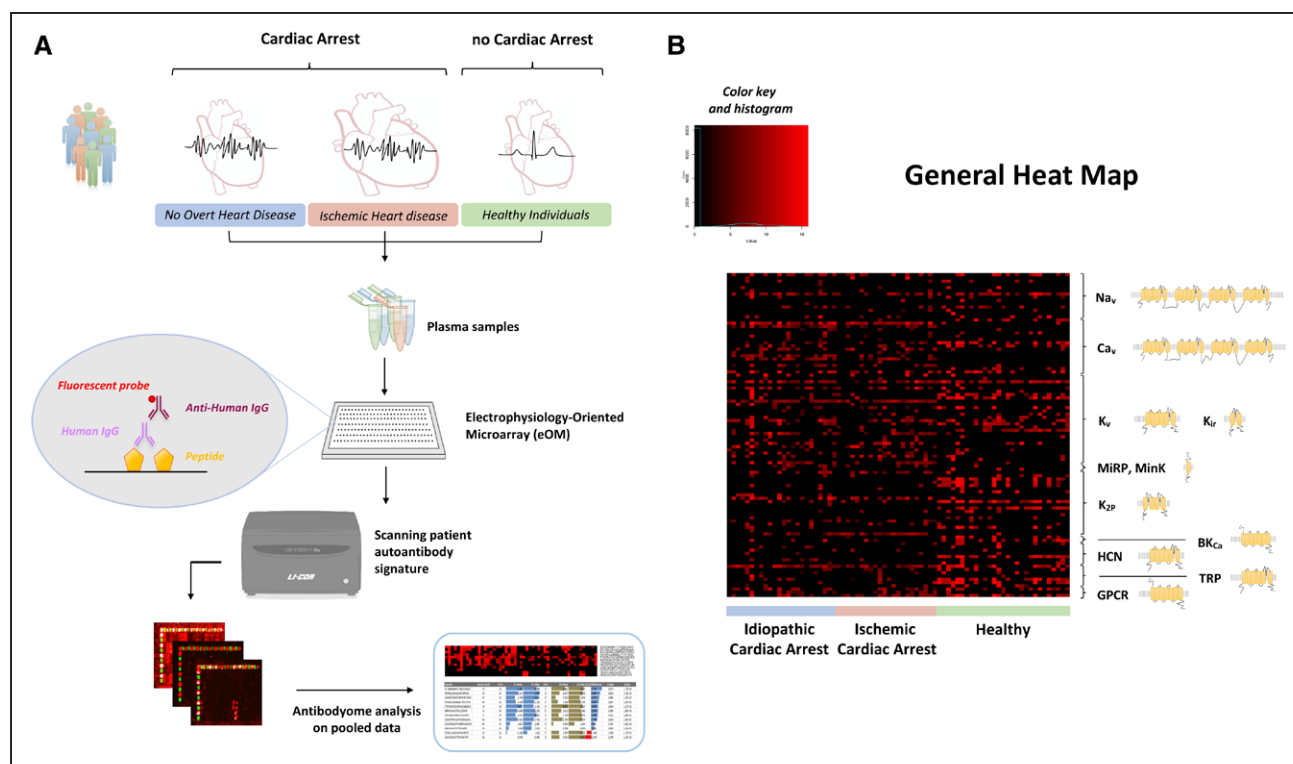


Figure 1. Schematic representation of the peptide microarray assay and general heat map of the immunoglobulin G (IgG) response profiles.

A, A step-by-step overview of the electrophysiology-oriented peptide microarray assay (eOM). More details are delineated in the Methods section. **B**, The heat map of 100 peptides analyzed for immunoreactivity across 74 plasma samples. Data were background corrected and log2 transformed. Fluorescence signal intensities of plasma samples from cardiac arrest groups and healthy individuals are shown. Peptide sequences on the y axis are plotted against the plasma samples on the x axis. BK_{Ca} indicates calcium-activated potassium channel; Ca_v, voltage-gated calcium channel; GPCR, G-protein-coupled receptor; HCN, hyperpolarization-activated cyclic nucleotide-gated cation channel; K_{ir}, inward rectifier potassium channel; K_{2p}, 2-pore domain potassium channel; K_v, voltage-gated potassium channel; MinK, minimal potassium channel subunit; MiRP, MinK-related peptides; Na_v, voltage-gated sodium channel; and TRP, transient receptor potential channel.

Whole-Cell Patch Clamp Recording in hiPSC-CMCs

Whole-cell patch clamp experiments were performed with the EPC-10 amplifier controlled by PATCHMASTER (HEKA Elektronik GmbH, Lambrecht, Germany) to record action potentials on spontaneously beating cardiomyocytes and the calcium current, I_{Ca} , at RT. The external patch solution used to record cardiac action potentials under current-clamp mode was composed of (mmol/L) 140 NaCl, 5 KCl, 1 MgCl₂, 10 HEPES, 1.8 CaCl₂, and 10 glucose (pH 7.4 adjusted with NaOH). Borosilicate glass pipettes (Harvard Apparatus, Holliston, MA) with tip resistances of 2 to 4 MΩ were filled with internal solution (mmol/L): 110 K⁺aspartate, 20 KCl, 1 MgCl₂, 5 Mg²⁺ATP, 0.1 Li⁺GTP, 10 HEPES, 5 Na⁺phosphocreatine, 0.05 EGTA (pH adjusted to 7.3 with KOH), and amphotericin B (200 μg/ml, AppliChem GmbH, Darmstadt, Germany). To record I_{Ca} currents, the following extracellular solution was used (mmol/L): 130 N-methyl-glucamine, 5 CsCl, 2 CaCl₂, 1.2 MgCl₂, 10 HEPES, and 5 glucose, titrated to pH 7.4 with CsOH. Borosilicate glass capillaries (Harvard Apparatus) with tip resistances of 5 to 8 MΩ were filled with internal solution composed of (mmol/L) 60 CsCl, 70 Cs-aspartate, 1 MgCl₂, 10 HEPES, 11 EGTA, and 5 Mg²⁺ATP, adjusted to pH 7.2 with CsOH. I_{Ca} was elicited with a holding potential of −80 mV, a 250-millisecond depolarization step to −40 mV (to isolate L- from T-type I_{Ca}), followed by 300-millisecond test pulses (at 0.2 Hz) to voltages between −50 and 30 mV in 10-mV increments.

Membrane potentials were corrected for a liquid junction potential of 12.4 mV. Whole-cell currents were low-pass filtered at 2.9 kHz and sampled at 4 kHz. The effect of purified anti-L-type voltage-gated calcium channel (Ca_v1.2) IgG (0.66 μg/mL; incubation, 24 hours) on hiPSC-CMCs was compared with cells under control condition. In addition, a peptide competition assay was performed to validate the specificity of the IgG. The antibodies were neutralized through preincubation with an excess of DFDNVLAAMMALFTVSTFEG peptide (Peptide Specialty Laboratories GmbH) that corresponds to the epitope recognized by the antibody (antibody-to-peptide ratio, 1:10). Patch clamp data analyses were performed with FITMASTER (HEKA Elektronik GmbH).

Statistical Analysis

The statistical analysis of microarray data was performed by statisticians from PEPperPRINT GmbH. The median intensity values of all peptides were background corrected and either log2 transformed for better visualization of the microarray data or variance-stabilizing normalized for 2-class *t* tests.¹⁵ Heat maps were generated with RStudio software (version 1.2.1335, RStudio, Inc, Boston, MA) to provide an overview of general similarities and differences of IgG responses between the groups. Pair-wise comparisons were based on 2-class *t* tests of the IgG responses of the idiopathic and ischemic cardiac arrest groups against the healthy group and on a 2-class *t* test of the idiopathic cardiac arrest against the

ischemic cardiac arrest group. The differences in fluorescence intensity between the respective groups were determined to reflect the overall intensity ratio. Two-sided values of $P < 0.05$ were considered the threshold for statistical significance of differential IgG responses. P values were further corrected for multiple testing error with 10% false discovery rate (FDR) using the Benjamini-Hochberg procedure.¹⁶ FDR is the expected proportion of type I errors in null hypothesis testing when multiple comparisons are conducted, as in the case of microarray assays. With a cutoff for significance $FDR < 0.10$, $< 10\%$ of the significant results would be false positives. All data were processed with the Statistical Utility for Microarray and Omics data software developed at the German Cancer Research Center in Heidelberg, Germany. To evaluate the efficiency of the panel of 100 peptides in the detection of idiopathic cardiac arrest and to develop IgG response-based classifiers for idiopathic cardiac arrest, least absolute shrinkage and selection operator (LASSO) logistic regression and random forest machine learning were applied. For LASSO logistic regression and random forest analysis, the 74 plasma samples were split into 2 parts: 85% of data for training and 15% for testing. Inverse hyperbolic transformation was applied for the training and test data separately. The algorithm was trained and fine-tuned to select the best/optimal model and further to predict and evaluate the model in the test data. Machine learning techniques were used to learn classifiers of idiopathic cardiac arrest, ischemic cardiac arrest, and healthy groups. Specifically, the 10-fold cross-validation LASSO with the R package glmnet was used (<https://glmnet.stanford.edu>).^{17,18} In addition, as a second orthogonal technique, random forests from the caret package was used (<https://CRAN.R-project.org/package=caret>).¹⁹ Accuracies based on confusion matrices were calculated for both the LASSO logistic regression and random forest methods. With regard to patient data, continuous variables are expressed as mean \pm SD, whereas nonnormally distributed variables are reported as median \pm interquartile range and categorical data in percentage. One-way ANOVA and the Tukey multiple-comparisons test were performed to compare the means of the continuous data. The χ^2 and Fisher exact tests were used for categorical variables. To compare the patch clamp data between groups, 1-way ANOVA was used, followed by the Tukey multiple-comparisons test. For nonnormally distributed data, the Kruskal-Wallis test was adopted, followed by the Dunn multiple-comparisons test. Statistical analyses were performed with GraphPad Prism 7 (GraphPad Software, Inc, La Jolla, CA), and a value of $P < 0.05$ was used for testing significance of potential clinical confounders.

RESULTS

Clinical Characteristics

Forty-five patients who survived a cardiac arrest (23 idiopathic, 22 ischemic) and 29 healthy subjects were enrolled in the present study (Table). Most cardiac arrest cases occurred out-of-hospital, whereas 4.3% of patients with idiopathic cardiac arrest and 27.3% of patients with ischemic cardiac arrest were in-hospital (Table). Patients with ischemic heart disease were

predominantly male, and the left ventricular ejection fraction was, as expected, reduced compared with the other groups. The median time between the echocardiographic examination and the cardiac arrest was 6 days (interquartile range, 8 days). There were no abnormalities on the ECG of the idiopathic cardiac arrest group (Table).^{20–22} Of note, case patients were deliberately selected on the basis of a normal ECG in line with the definition of idiopathic cardiac arrest. Just as cardiac ion channel mutation carriers can have a normal ECG, the presence of potentially arrhythmogenic autoantibodies and the absence of ECG features are not mutually exclusive. Provocative testing (pharmacological challenge with epinephrine or procainamide) was performed in 8.7% of patients with idiopathic cardiac arrest and showed no electrocardiographic patterns diagnostic of long-QT syndrome, catecholaminergic polymorphic VT, or Brugada syndrome. Sixty-five percent of patients with unexplained cardiac arrest were subjected to genetic testing that yielded no disease-causing mutation.

Autoantibody Response Profiles

In the present study, a peptide microarray encompassing 100 epitopes corresponding to 33 different cardiac autoantigens was used to determine specific IgG responses in patients. The median time elapsed between the plasma sampling and the cardiac arrest event was 4 years (interquartile range, 10 years). As visualized on the general heat map, plasma samples from each patient recognized different sets of peptides (Figure 1B). Most essentially, the existence of to-date unknown antibodies was detected in both patients and healthy individuals. A common IgG response against the 2 peptides AEVDVRVDSHFTSIPESFWWA ($K_v1.7$ protein) and PTGPNATESVQPMEGQEDEG (TRPV2 protein) could be found in nearly all samples.

Candidate IgG Biomarkers of Cardiac Arrest

Autoantibody signatures for patients with cardiac arrest were identified. First, the differential IgG responses were evaluated individually with a univariate selection approach and ranked according to the differences that reflect the overall intensity ratio between the idiopathic cardiac arrest, ischemic cardiac arrest, and healthy groups. By controlling the FDR at 10%, we identified 16 statistically significant differential IgG responses (Figure 2). To estimate the adjusted association of each IgG in the presence of the others and their joint IgG response, it is critical to analyze the differential IgG results with a multivariable approach. A multivariable modeling of biomarkers was therefore performed that included 10-fold cross-validation

Table. Clinical Characteristics of the Study Population*

	Idiopathic Cardiac Arrest	Ischemic Cardiac Arrest	Healthy	P Value [§]
Patients, n	23	22	29	
Age, mean±SD, y	53.5±14.7	55.9±10.9	48.6±14.9	0.15
Sex, %				0.30
Male	56.5	72.7	51.7	
Female	43.5	27.3	48.3	
BMI, mean±SD, kg/m ²	29.9±6.2	29.6±6.6	27.9±5.0	0.42
Electrocardiographic parameters				
Rhythm, % sinus rhythm	100	86.4	100	0.10
Heart rate, mean±SD, bpm	81.7±14.0	71.8±14.4	75.5±12.2	0.09
PR, mean±SD, ms	168.1±21.1	177.5±34.3	155.6±13.4	0.02
QRS, mean±SD, ms	95.7±10.3	96.6±15.7	87.3±9.3	0.01
QT, mean±SD, ms	383.5±26.7	384.4±40.2	380.0±27.8	0.88
Corrected QT _{Bazett} [†] , mean±SD, ms	445.1±41.4	417.7±35.5	422.9±20.7	0.03
Corrected QT _† [‡] , mean±SD, ms	421.5±30.4	405.5±32.0	407.2±17.2	0.14
Corrected QT _† [§] , mean±SD, ms	429.0±35.5	408.0±33.4	411.2±18.2	0.06
Echocardiographic parameters				
LVEF, mean±SD, %	60.0±5.3	47.3±16.5	62.5±3.6	<0.01
LVEDD, mean±SD, mm	47.2±6.3	51.6±12.6	47.7±5.4	0.85
IVSD, mean±SD, mm	9.4±2.2	10.3±1.9	9.8±1.7	0.46
PWD, mean±SD, mm	9.2±1.8	9.0±1.2	8.7±1.8	0.80

BMI indicates body mass index; IVSD, interventricular septum diameter; LVEDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; and PWD, posterior wall diameter.

*Values are mean±SD or number (percent). Age refers to the age at study enrollment.

[†]The QT interval was corrected with the Hodges formula [$QT_c = QT + 1.75 \times (\text{heart rate} - 60)$] for QRS <120 milliseconds.²⁰

[‡]The QT interval was corrected with the method by Rautaharju et al²¹ [$QT_c = QT \times (120 + \text{heart rate}) / 180$] for QRS <120 milliseconds. In the case of QRS ≥120 milliseconds in the ischemic cardiac arrest group, the following equation was used to adjust the QT interval: $QT_c = QT - 155 \times (60 / \text{heart rate} - 1) - 0.93 \times (QRS - 139) + k(k_{\text{male}} = -22; k_{\text{female}} = -34)$.²² In the presence of atrial fibrillation, a weighted average modification of the Bazett formula was applied [$QT_c = QT / (RR_{\text{mod}})^{1/2}$].¹¹

[§]P values comparing idiopathic cardiac arrest vs ischemic cardiac arrest vs healthy group (ANOVA for normally distributed variables and Kruskal-Wallis test for nonnormally distributed data).

LASSO logistic regression and random forest machine learning method as second orthogonal technique. Briefly, the LASSO logistic regression considers IgG responses with nonzero coefficients as potential biomarkers, and the random forest method identifies them by ranking the IgG response according to the variable importance. In the present study, both algorithms identified different sets of IgG response patterns associated with the diagnosis of idiopathic cardiac arrest compared with the ischemic cardiac arrest and healthy group (Figure II in the Data Supplement). The accuracy to determine the discriminatory performance of each method in determining idiopathic cardiac arrest was also evaluated. Both methods performed similarly. With an accuracy of 83% and 86%, the peptide-based microarray assay presents a high potential to distinguish idiopathic cardiac arrest from ischemic cardiac arrest and healthy control subjects respectively. Taken together, 1 single autoantibody was significantly upregulated in patients with idiopathic cardiac arrest compared with the ischemic

cardiac arrest and healthy groups ($P=0.002$), persisting after Benjamini-Hochberg correction (FDR, 0.007) and multivariable analyses (LASSO logistic regression and random forest): the autoantibody targeting the third extracellular loop of domain III (DIIIIE3) of $Ca_v1.2$, a pore-forming region of the voltage-gated calcium channel (DFDNVLAAMMALFTVSTFEG; Figure 2). After adjustment with the Benjamini-Hochberg post hoc analysis and multivariable modeling (consistent across both methods), autoantibodies recognizing the second pore of the TREK1 (TWIK-related potassium channel 1) protein (DYVAGGSDIEYLDYKPVVW) were strongly associated with cardiac arrest of ischemic origin and healthy control subjects ($P<0.001$; FDR <0.001; Figure 2). Plasma samples from healthy individuals presented high IgG responses with a complex autoantibody pattern against cardiac ion channels. Overall, marked IgG reactivities were observed for potassium channels and other sequences of the $Ca_v1.2$ channel and the β_1 -adrenergic receptor compared with the cardiac arrest population (Figure 2).

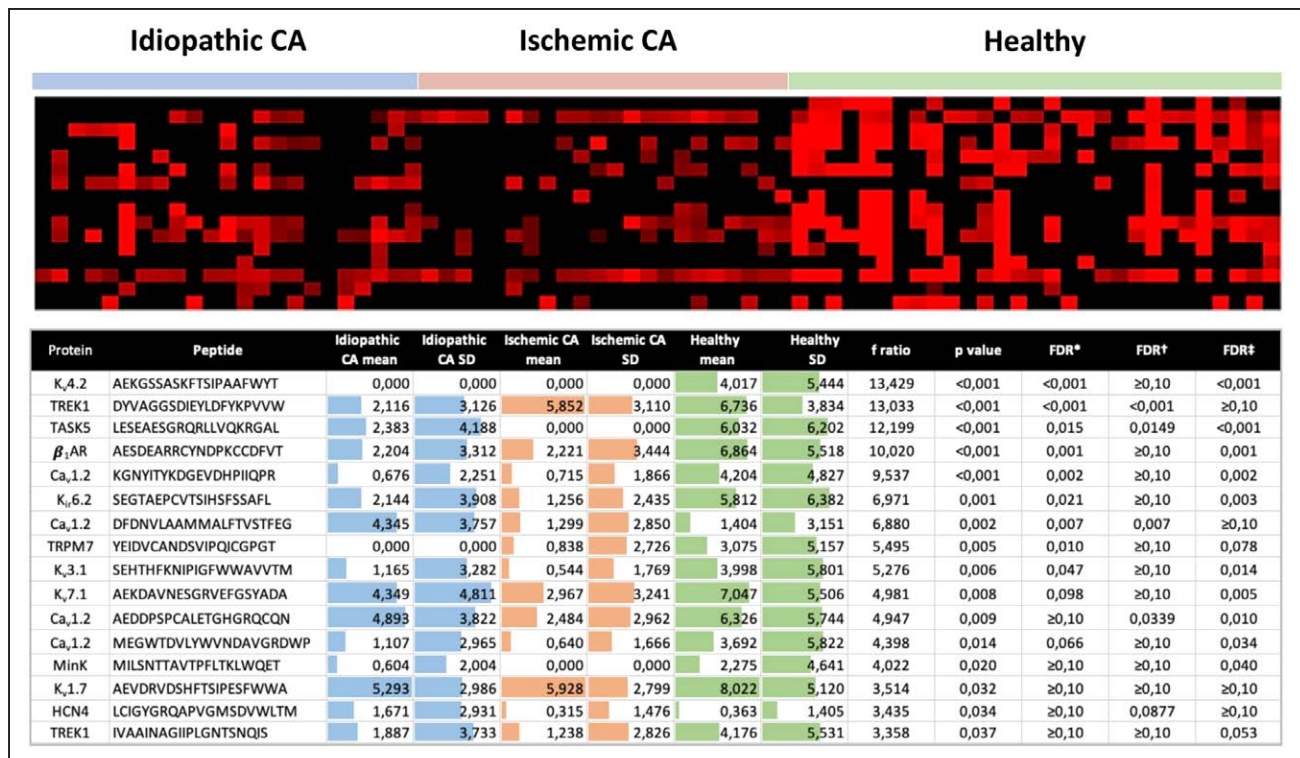


Figure 2. Cardiac arrest (CA)-specific antibody reactivities in plasma samples.

Listed are the peptides that demonstrated the highest immunoglobulin G (IgG) response in the idiopathic CA, ischemic CA, and healthy groups. The ranking was based on differences that reflect the overall intensity ratio between the groups. ANOVA compared the mean IgG responses from patients with idiopathic CA and ischemic CA and healthy control subjects. Reported are the respective *P* values and *f* ratio. The corresponding excerpt of the heat map is shown with the peptide sequences on the y axis plotted against the plasma samples on the x axis. *False discovery rate (FDR) correction for idiopathic CA vs healthy. †FDR correction for idiopathic CA vs ischemic CA. ‡FDR correction for ischemic CA vs healthy.

Purification and Functional Characterization of the Candidate IgG

Next, the electrophysiological effects of anti-Ca_v1.2 antibody targeting the DIIIE3 domain were explored on hiPSC-CMCs. Figure 3 shows representative action potential and *I*_{Ca} current traces. The anti-Ca_v1.2 antibody significantly reduced the action potential duration at 90% repolarization by 2.8-fold (control, 1178.0±169.3 milliseconds versus anti-Ca_v1.2 IgG, 416.7±22.2 milliseconds; *P*<0.001; Figure 3A) and depolarized the maximum diastolic potential (control, -71.8±3.2 mV versus anti-Ca_v1.2 IgG, -50.3±4.9 mV; *P*=0.001). Of 13 cells, the antibody induced an episode of alternans, a typical arrhythmia substrate. The *I*_{Ca} current was recorded in hiPSC-CMCs. In accordance with the action potential duration shortening, the purified anti-Ca_v1.2 antibody decreased *I*_{Ca} by 2-fold at the potential of -10 mV (*P*<0.001; Figure 3B). In addition, the presence of the blocking peptide corresponding to the DIIIE3 sequence abolished the effect of the antibody on cardiac action potential and *I*_{Ca}, thus confirming the IgG-specific response in vitro (Figure 3). The cell capacitance did not differ between groups (control, 17.0±1.6 pF; anti-Ca_v1.2 IgG, 24.4±3.9 pF; anti-Ca_v1.2 IgG+blocking peptide, 21.0±3.4 pF; *P*=0.20). There was

no autoantibody-associated shift of the half-maximal activation voltage (Figure III in the Data Supplement).

DISCUSSION

The present peptide microarray study compared the autoantibody-binding profiles of patients with cardiac arrest. Hitherto unknown autoantibodies targeting extracellular sequences of cardiac ion channels were detected. The study identified an autoantibody signature specific to patients with cardiac arrest.

In previous studies, autoantibodies directed against cardiac antigens have been identified and linked to both tachyarrhythmias and bradyarrhythmias.^{7,23} For example, circulating autoantibodies recognizing the β₁-adrenergic receptor can be detected in 30% to 50% of patients with dilated cardiomyopathy and are independent predictors of VT and sudden cardiac death.²⁴ A high prevalence of anti-β₂-adrenergic receptor autoantibodies was found in patients with ventricular arrhythmias.²⁵ With cardiac ion channels as targets, autoantibodies against the KCNH2 (hERG) channel have been associated with the long-QT syndrome in a patient with recurrent torsades de pointes tachycardia.²⁶ Finally, autoantibodies targeting the L-type calcium channel were found in patients with dilated cardiomyopathy and have

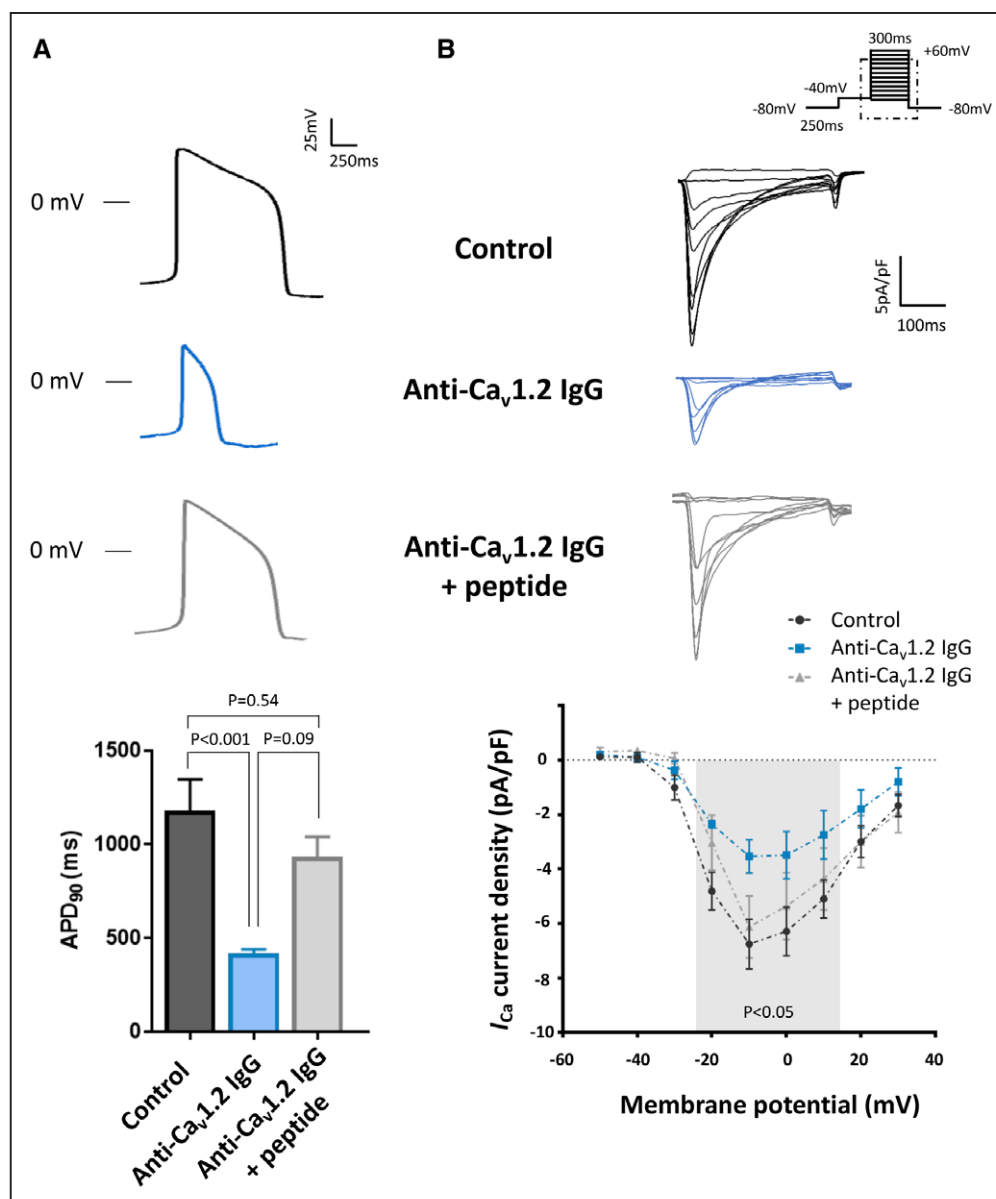


Figure 3. Whole-cell patch clamp recordings in human induced pluripotent stem cell-derived ventricular cardiomyocytes (hiPSC-CMCs).

A, Representative action potential traces. The action potential duration was determined at 90% repolarization (APD₉₀) for control cells (1178.0 ± 169.3 milliseconds; n=16), in the presence of anti-L-type voltage-gated calcium channel (Ca_v1.2) immunoglobulin G (IgG; 416.7 ± 22.2 milliseconds; n=13) vs anti-Ca_v1.2 IgG+blocking peptide (933.3 ± 107.1 milliseconds; n=6). **B**, Representative I_{Ca} (control, 16.2 pF; anti-Ca_v1.2 IgG, 22.5 pF; anti-Ca_v1.2 IgG+blocking peptide, 21.3 pF) current responses to a voltage step from -50 to 30 mV (see outlined voltage step stimulation protocol). **Bottom**, I-V relationships of control cells (n=9) and in the presence of anti-Ca_v1.2 IgG (n=8) and anti-Ca_v1.2 IgG+blocking peptide (n=5) are presented. I_{Ca} currents are expressed as densities normalized to cell capacitance. The respective mean ± SEM values are displayed. P < 0.05, control vs anti-Ca_v1.2 IgG. All measurements were performed at room temperature.

been linked to a higher incidence of VT and cardiac arrest.²⁷ Of note, these latter autoantibodies recognize an epitope corresponding to an intracellular sequence of the L-type calcium channel. How immunoglobulins can access to and interact with an intracellular target remains uncertain.

The present study characterized the autoantibody signature associated with cardiac arrest. Autoantibodies may perturb the flow of ions through the channels either by physically obstructing the conduction through the pore or by acting as an agonist (ligand mimicry or via allosteric modulation).^{11,12} As genetic mutations

have taught us, a loss or gain of function of a certain channel can result in cardiac arrhythmias and presents the basis for a number of arrhythmia syndromes. With this hypothesis in mind, interest in screening patients for autoantibodies against a variety of antigenic epitopes of cardiac ion channels has become evident. The analyses demonstrated distinct patterns of autoantibody profiles. Using the FDR method, LASSO logistic regression, and random forest, the study identified IgGs targeting the peptide DFDNVLAAAMMALFTVST-FEG (Ca_v1.2 channel) as a potential biomarker linked to idiopathic cardiac arrest with accuracies of 83%

and 86%. Antibodies targeting the DYVAGGSDIEYLD-FYKPVVW (TREK1 channel) sequence could be distinguished across both multivariable modeling methods and had a significant univariable association with the healthy and the cardiac arrest population with underlying ischemic heart disease. Because calcium channels form depolarizing inward currents that are critical for the plateau phase of the action potential, it is not surprising that perturbations of their function can lead to cardiac arrhythmias.²⁸ The target peptide DIIIE3 is localized at the ion selectivity and permeability filter of the calcium channel, a site where a missense mutation (CACNA1C-E1115K) has been related to the Brugada syndrome and sudden cardiac death.^{29,30} In the present study, the existence of an autoantibody specifically targeting the pore region of the calcium channel is described. Moreover, the DIIIE3-targeted anti-Ca_v1.2 antibodies reduce action potential duration by their calcium channel-inhibiting effect in hiPSC-CMCs and depolarize the membrane potential, both arrhythmogenic substrates. Two-pore domain potassium channels are essential in mediating background outward currents in cardiomyocytes. Notably, TREK1 channels are important modulators of the repolarization phase of the action potential, and alterations in their function have been linked to VT.³¹ Autoantibodies targeting TREK1 have not been reported before. Future studies are warranted to determine the role of anti-TREK1 antibodies.

In the present study, healthy individuals demonstrated a complex profile of IgG autoantibodies directed against several cardiac antigens. The abundance and diversity of circulating autoantibodies imply that they may carry out some important but as yet unrecognized function. Boyden³² first introduced the term natural autoantibodies to describe the immune system, recognizing self-constituents in healthy persons. Natural autoantibodies are thought to play an important physiological role, notably in the maintenance of tissue homeostasis through the adaptive clearance of tissue and cell debris after degradation.^{33,34} Healthy individuals in the present study presented high IgG reactivities to the peptide sequence KGNYYTYKDGEVDHPHIIQPR of the L-type calcium channel, only 7 amino acids upstream of the DIIIE3 autoantibody target of the idiopathic cardiac arrest population (DFDNVLAAMMALFTVSTFEG). The close proximity of the targets suggests the possibility of a competitive antagonistic interaction between both autoantibodies. Future studies are needed to verify this hypothesis attributing a protective role of autoantibodies directed against KGNYYTYKDGEVDHPHIIQPR. Contrary to expectation, a stronger IgG response directed against the second extracellular loop of the β_1 -adrenergic receptor was found in the healthy group. Anti- β_1 -adrenergic receptor autoantibodies have been described mainly in the context of dilated cardiomyopathies with proarrhythmic properties.²⁵ However,

they also have been detected in the healthy population, with the frequency of occurrence increasing with age.³⁵ Their role in healthy individuals is yet to be defined.

The advantage with peptide microarrays is the high-throughput screening of plasma samples for IgG reactivities against many peptides simultaneously. However, the findings of this study have to be seen in light of some limitations. Limitations of the assay are the failure to consider conformational epitopes, that is, the natively folded protein structure, and the respective affinity of the antibodies. Nevertheless, the identified antibody targeting the extracellular calcium channel epitope has shown functionality in blocking channel function. The role of the other autoantibodies detected in each patient population has not been investigated. Although patients with autoimmune disorders were excluded from the study, an assessment of patients' signs and symptoms suggestive of autoimmune diseases was not performed after study inclusion. The number of idiopathic cardiac arrest cases in this study was relatively small, an inherent problem in studies with stringent inclusion and exclusion criteria for a condition for which plasma samples are rarely collected. Larger patient cohorts with follow-up studies will be important to better understand the peripheral antibody repertoire against cardiac ion channels. Most important, all the plasma samples of patients were obtained after the index event. Consequently, it cannot be excluded that the identified autoantibodies could have been generated as a result of cardiac arrest rather than being the cause of cardiac arrest. However, if cardiac arrest were to induce an autoimmune response, one would likely have expected to find similar IgG response profiles in cardiac arrest of ischemic and idiopathic origin. How the autoimmune mechanism is initiated and regulated remains largely unknown. Future studies are necessary to differentiate the role of the autoantibodies in cardiac arrest, that is, to distinguish between preceding autoantibodies (and thus contributing to cardiac arrest) and autoantibodies generated in response to cardiac arrest. In addition, subsequent studies addressing the (protective?) role of autoantibodies targeting cardiac ion channels in the healthy will be of particular interest. A further limitation of the study is that only survivors of cardiac arrest were considered; that is, decedents were excluded from the autoantibody screening. The results are therefore subject to survivor bias. Nonetheless, the *in vitro* proarrhythmic properties of anti-Ca_v1.2 autoantibodies from patients with idiopathic cardiac arrest indicate that the IgG may have disease relevance.

Conclusions

The findings of the present study suggest that a distinct immunosignature distinguishes patients with aborted cardiac arrest from healthy control subjects.

ARTICLE INFORMATION

Received October 17, 2019; accepted April 2, 2020.

The Data Supplement is available with this article at <https://www.ahajournals.org/doi/suppl/10.1161/CIRCULATIONAHA.119.044408>.

Correspondence

Jin Li, MD, Institute of Biochemistry and Molecular Medicine, University of Bern, Buehlstrasse 28, 3012 Bern, Switzerland; or Department of Cardiology, Lausanne University Hospital, Rue du Bugnon 46, 1011 Lausanne, Switzerland. Email jin.li@ibmm.unibe.ch or jin.li@chuv.ch

Affiliations

Institute of Physiology (A.M.) and Institute of Biochemistry and Molecular Medicine (J.L.), University of Bern, Switzerland. Montreal Heart Institute, Université de Montréal, QC, Canada (J.C.T., D.B.). Division of Immunology and Allergy (C.R.) and Department of Cardiology (J.L.), Lausanne University Hospital, Switzerland.

Acknowledgments

The authors thank the excellent support provided by the team of PEPperPRINT GmbH, in particular Gregor Jainta, Dr Eric Dyrz, and Dr Yuvaraj Mahendran.

Sources of Funding

This work was supported by grants from the Swiss National Science Foundation (Ambizione PZ00P3_173961 to Dr Li) and from the Jubiläumsstiftung of SwissLife (to Dr Li).

Disclosures

Dr Tardif reports grants from Amarin, AstraZeneca, Ionis, and Regeneron; grants, personal fees, and other from DalCor; and grants and personal fees from Pfizer, Sanofi, and Servier. The other authors report no conflicts.

Supplemental Materials

Data Supplement Figures I–III
Data Supplement Table I

REFERENCES

- Benjamin EJ, Muntner P, Alonso A, Bittencourt MS, Callaway CW, Carson AP, Chamberlain AM, Chang AR, Cheng S, Das SR, et al; American Heart Association Council on Epidemiology and Prevention Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics—2019 update: a report from the American Heart Association. *Circulation*. 2019;139:e56–e528. doi: 10.1161/CIR.0000000000000659
- Waldmann V, Bougouin W, Karam N, Dumas F, Sharifzadehgan A, Gandjbakhch E, Algalarrondo V, Narayanan K, Zhao A, Amet D, et al; Paris-SDEC Investigators. Characteristics and clinical assessment of unexplained sudden cardiac death in the real-world setting: focus on idiopathic ventricular fibrillation. *Eur Heart J*. 2018;39:1981–1987. doi: 10.1093/eurheartj/ehy098
- Priori SG, Blomström-Lundqvist C. 2015 European Society of Cardiology guidelines for the management of patients with ventricular arrhythmias and the prevention of sudden cardiac death summarized by co-chairs. *Eur Heart J*. 2015;36:2757–2759. doi: 10.1093/eurheartj/ehv445
- Hayashi M, Shimizu W, Albert CM. The spectrum of epidemiology underlying sudden cardiac death. *Circ Res*. 2015;116:1887–1906. doi: 10.1161/CIRCRESAHA.116.304521
- Krahn AD, Healey JS, Chauhan V, Birnie DH, Simpson CS, Champagne J, Gardner M, Sanatani S, Xner DV, Klein GJ, et al. Systematic assessment of patients with unexplained cardiac arrest: Cardiac Arrest Survivors With Preserved Ejection Fraction Registry (CASPER). *Circulation*. 2009;120:278–285. doi: 10.1161/CIRCULATIONAHA.109.853143
- Modi S, Krahn AD. Sudden cardiac arrest without overt heart disease. *Circulation*. 2011;123:2994–3008. doi: 10.1161/CIRCULATIONAHA.110.981381
- Lee HC, Huang KT, Wang XL, Shen WK. Autoantibodies and cardiac arrhythmias. *Heart Rhythm*. 2011;8:1788–1795. doi: 10.1016/j.hrthm.2011.06.032
- von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP; STROBE Initiative. Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. *BMJ*. 2007;335:806–808. doi: 10.1136/bmj.39335.541782.AD
- Al-Khatib SM, Stevenson WG, Ackerman MJ, Bryant WJ, Callans DJ, Curtis AB, Deal BJ, Dickfeld T, Field ME, Fonarow GC, et al. 2017 AHA/ACC/HRS guideline for management of patients with ventricular arrhythmias and the prevention of sudden cardiac death: a report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines and the Heart Rhythm Society. *Circulation*. 2018;138:e272–e391. doi: 10.1161/CIR.0000000000000549
- Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, et al; ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*. 2015;17:405–424. doi: 10.1038/gim.2015.30
- Li J, Seyler C, Wiedmann F, Schmidt C, Schweizer PA, Becker R, Katus HA, Thomas D. Anti-KCNQ1 K⁺ channel autoantibodies increase I_{Ks} current and are associated with QT interval shortening in dilated cardiomyopathy. *Cardiovasc Res*. 2013;98:496–503. doi: 10.1093/cvr/cvt046
- Li J, Maguy A, Duverger JE, Vigneault P, Comtois P, Shi Y, Tardif JC, Thomas D, Nattel S. Induced KCNQ1 autoimmunity accelerates cardiac repolarization in rabbits: potential significance in arrhythmogenesis and antiarrhythmic therapy. *Heart Rhythm*. 2014;11:2092–2100. doi: 10.1016/j.hrthm.2014.07.040
- Kolaskar AS, Tongaonkar PC. A semi-empirical method for prediction of antigenic determinants on protein antigens. *FEBS Lett*. 1990;276:172–174. doi: 10.1016/0014-5793(90)80535-q
- Yao B, Zhang L, Liang S, Zhang C. SVMTrIP: a method to predict antigenic epitopes using support vector machine to integrate tripeptide similarity and propensity. *PLoS One*. 2012;7:e45152. doi: 10.1371/journal.pone.0045152
- Huber W, von Heydebreck A, Sülthmann H, Poustka A, Vingron M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics*. 2002;18(suppl 1):S96–S104. doi: 10.1093/bioinformatics/18.suppl_1.s96
- Benjamini YH, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc*. 1995;57:289–300.
- Friedman J, Hastie T, Tibshirani R. Regularization paths for generalized linear models via coordinate descent. *J Stat Softw*. 2010;33:1–22.
- Simon N, Friedman J, Hastie T, Tibshirani R. Regularization paths for Cox's proportional hazards model via coordinate descent. *J Stat Softw*. 2011;39:1–13. doi: 10.18637/jss.v039.i05
- Kuhn M. Building predictive models in R using the caret package. *J Stat Softw*. 2008;28:1–26.
- Chiladakis J, Kalogeropoulos A, Arvanitis P, Koutsogiannis N, Zagli F, Alexopoulos D. Heart rate-dependence of QTc intervals assessed by different correction methods in patients with normal or prolonged repolarization. *Pacing Clin Electrophysiol*. 2010;33:553–560. doi: 10.1111/j.1540-8159.2009.02657.x
- Rautaharju PM, Mason JW, Akiyama T. New age- and sex-specific criteria for QT prolongation based on rate correction formulas that minimize bias at the upper normal limits. *Int J Cardiol*. 2014;174:535–540. doi: 10.1016/j.ijcard.2014.04.133
- Rautaharju PM, Zhang ZM, Prineas R, Heiss G. Assessment of prolonged QT and JT intervals in ventricular conduction defects. *Am J Cardiol*. 2004;93:1017–1021. doi: 10.1016/j.amjcard.2003.12.055
- Lazzerini PE, Capecci PL, Laghi-Pasini F, Boutjdir M. Autoimmune channelopathies as a novel mechanism in cardiac arrhythmias. *Nat Rev Cardiol*. 2017;14:521–535. doi: 10.1038/nrcardio.2017.61
- Fukuda Y, Miyoshi S, Tanimoto K, Oota K, Fujikura K, Iwata M, Baba A, Hagiwara Y, Yoshikawa T, Mitamura H, et al. Autoimmunity against the second extracellular loop of beta(1)-adrenergic receptors induces early afterdepolarization and decreases in K-channel density in rabbits. *J Am Coll Cardiol*. 2004;43:1090–1100. doi: 10.1016/j.jacc.2003.09.057
- Chiale PA, Rosenbaum MB, Elizari MV, Hjalmarson A, Magnusson Y, Wallukat G, Hoebeke J. High prevalence of antibodies against beta 1- and beta 2-adrenoceptors in patients with primary electrical cardiac abnormalities. *J Am Coll Cardiol*. 1995;26:864–869. doi: 10.1016/0735-1097(95)00262-2
- Nakamura K, Katayama Y, Kusano KF, Haraoka K, Tani Y, Nagase S, Morita H, Miura D, Fujimoto Y, Furukawa T, et al. Anti-KCNH2

antibody-induced long QT syndrome: novel acquired form of long QT syndrome. *J Am Coll Cardiol*. 2007;50:1808–1809. doi: 10.1016/j.jacc.2007.07.037

27. Xiao H, Wang M, Du Y, Yuan J, Cheng X, Chen Z, Zou A, Wei F, Zhao G, Liao YH. Arrhythmogenic autoantibodies against calcium channel lead to sudden death in idiopathic dilated cardiomyopathy. *Eur J Heart Fail*. 2011;13:264–270. doi: 10.1093/eurjhf/hfq198
28. Grant AO. Cardiac ion channels. *Circ Arrhythm Electrophysiol*. 2009;2:185–194. doi: 10.1161/CIRCEP.108.789081
29. Burashnikov E, Pfeiffer R, Barajas-Martinez H, Delpón E, Hu D, Desai M, Borggrefe M, Häissaguerre M, Kanter R, Pollevick GD, et al. Mutations in the cardiac L-type calcium channel associated with inherited J-wave syndromes and sudden cardiac death. *Heart Rhythm*. 2010;7:1872–1882. doi: 10.1016/j.hrthm.2010.08.026
30. Ye D, Tester DJ, Zhou W, Papagiannis J, Ackerman MJ. A pore-localizing CACNA1C-E1115K missense mutation, identified in a patient with idiopathic QT prolongation, bradycardia, and autism spectrum disorder, converts the L-type calcium channel into a hybrid nonselective monovalent cation channel. *Heart Rhythm*. 2019;16:270–278. doi: 10.1016/j.hrthm.2018.08.030
31. Decher N, Kiper AK, Rinné S. Stretch-activated potassium currents in the heart: focus on TREK-1 and arrhythmias. *Prog Biophys Mol Biol*. 2017;130(pt B):223–232. doi: 10.1016/j.pbiomolbio.2017.05.005
32. Boyden S. Cellular recognition of foreign matter. *Int Rev Exp Pathol*. 1963;2:311–356.
33. Avrameas S, Selmi C. Natural autoantibodies in the physiology and pathophysiology of the immune system. *J Autoimmun*. 2013;41:46–49. doi: 10.1016/j.jaut.2013.01.006
34. Nagele EP, Han M, Acharya NK, DeMarshall C, Kosciuk MC, Nagele RG. Natural IgG autoantibodies are abundant and ubiquitous in human sera, and their number is influenced by age, gender, and disease. *PLoS One*. 2013;8:e60726. doi: 10.1371/journal.pone.0060726
35. Liu HR, Zhao RR, Zhi JM, Wu BW, Fu ML. Screening of serum autoantibodies to cardiac beta1-adrenoceptors and M2-muscarinic acetylcholine receptors in 408 healthy subjects of varying ages. *Autoimmunity*. 1999;29:43–51. doi: 10.3109/08916939908995971